PROTEOLYTIC DEGRADATION OF HISTONES AND SITE OF CLEAVAGE IN HISTONE F2al AND F3

W. F. BRANDT, L. BÖHM and C. VON HOLT

Department of Biochemistry, University of Cape Town, South Africa

Received 12 December 1974

1. Introduction

Histones are susceptible to proteolytic degradation during their isolation [1,2]. This degradation can be minimized by protease inhibitors like diisopropylphosphofluoridate and by performing all operations at low temperatures [1]. Subsequently it has been shown that a trypsin-like protease is closely associated with isolated chromatin [2–4]. This enzyme is co-extracted with histones in dilute acid and may autolyse such a preparation near neutral pH [2,5]. The enzyme has its optimal activity at pH 8 with nucleoprotein or free histones as substrate [2,3]. The disc electrophoretic pattern of the histone fraction isolated from degraded nucleoprotein reveals that only a limited number of fragments are formed [1,2], and that the 5 histone groups show different susceptibility to the proteolysis [2]. Although the enzyme has been partially purified [3], its specificity has not yet been determined.

We now wish to report the purification and characterization of two histone degradation products isolated from cycad pollen nucleoprotein. The results of the determination of the N-terminal sequence of these fragments have allowed us to identify them as breakdown products of histone F3 and F2al respectively. A number of interesting features of the cleavage sites became apparent. Possible biological implications are briefly discussed.

2. Materials and methods

Nucleoprotein was isolated from cycad pollen (*Encephalartos caffer*) by rupturing the pollen grains in 0.1 M NaCl–0.01 sodium citrate–0.01 $Na_2S_2O_5$ – 0.01 M EDTA in a cell homogenizer (B. Braun, model MSK [6] using 0.5 mm glass bals. The homogenate was centrifuged at 500 *g* for 4 min. The nucleoprotein in the supernatant was recovered by centrifugation at 30 000 *g* for 10 min. The resulting pellet was washed in the saline solution by repeated homogenization and centrifugation [7]. Nucleoprotein still present in the washed '500 *g*' pellet, which mainly consisted of pollen envelopes, was recovered by extraction with 2 M NaCl [8].

Histones were extracted from nucleoprotein preparations by 0.25 N HCl extraction [1]. Histones were recovered from the extract by acetone precipitation [9]. Arginine-rich histones were selectively extracted from nucleoprotein with 0.25 N HCl in 80% ethanol [9] and then recovered by adding 5 vols of cold acetone. Gel electrophoresis, the determination of the amino acid composition and the determination of amino acid sequences were performed as previously published [10]. All other details to experimental methods are given in legends to figures and tables.

3. Results and discussion

During our studies on the isolation and properties of histones from cycad pollen we noticed that the histones from this source are very prone to proteolytic degradation even in the presence of bisulphite [1]. Nearly undegraded cycad histones (fig.1, gel 2) could, however, be isolated by acid extraction from crude nucleoprotein when precautions were taken to minimize proteolytic degradation, i.e. isolation in the shortest possible time at low temperatures.

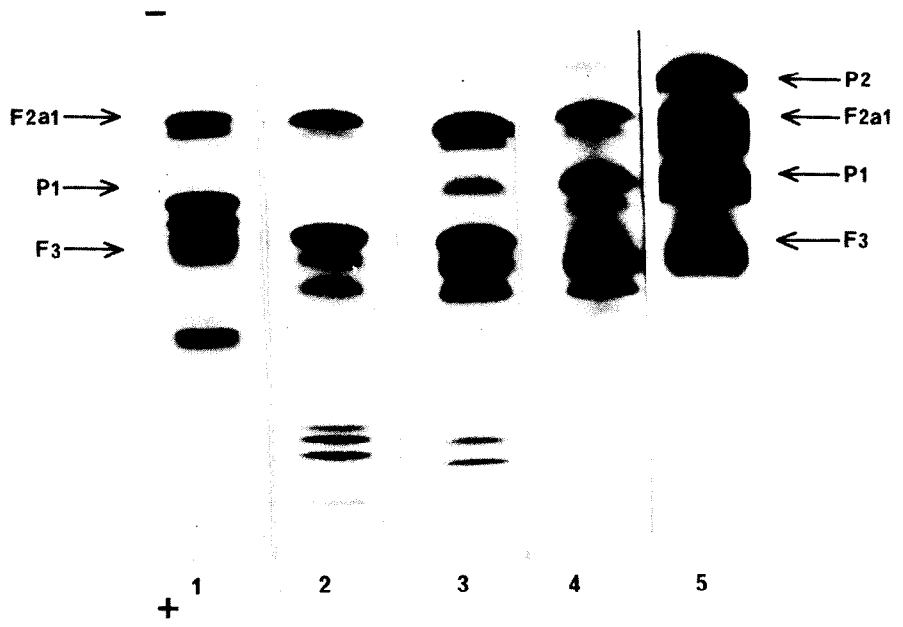

Fig.1. Disc electrophoretic pattern of degraded cycad pollen histones. Gel 1: calf thymus histones (20 μg), gel 2: undegraded cycad pollen (20 μg), gel 3 and 4: progressively longer degraded cycad histone, gel 5: ethanol–HCl extract of degraded cycad histones containing fragments P1 and P2. All samples were dissolved in 6 M urea–1% mercaptoethanol and run on 15% acrylamide gels for 3.5 hr. No attempt was made to apply equivalent amounts of protein to gels.

Nucleoprotein that had been isolated by a longer procedure for example by 2 M NaCl extraction, yielded partially degraded histones. From fig.1, gel 3, it becomes apparent that both histone F3 and the slowest moving histone fraction are partially degraded. The degradation of histones even seems to continue at low temperatures. After a storage of 4 months at −20°C histone F3 is nearly completely degraded (fig.1, gel 4). A similar degree of degradation is obtained on incubating nucleoprotein in 0.01 M Tris buffer pH 8 for a few hours at room temperature.

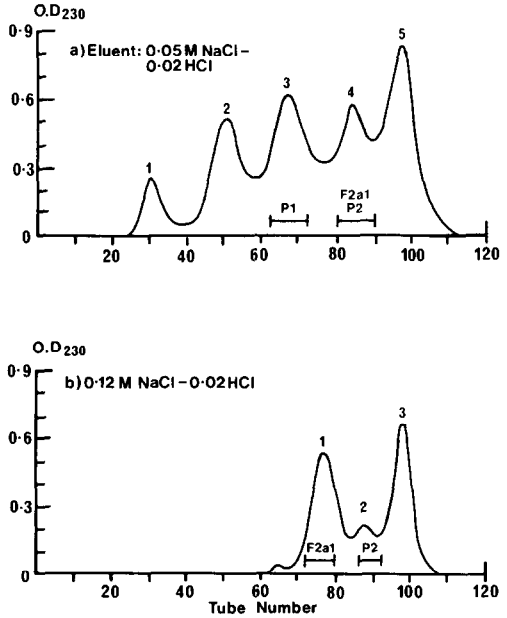

Fig.2. Purification of fragment P1 and P2 by exclusion chromatography on Biogel P-60. a) Elution pattern of the ethanol–HCl extract of degraded nucleoprotein. Peak 5 is due to urea and mercaptoethanol. b) Rechromatography of Peak 4 on same column, but instead of using 0.05 N NaCl–0.02 N HCl, 0.12 N NaCl–0.02 N HCl was used. Peak 3 is due to urea and mercaptoethanol. The column dimensions were 2.5 × 100 cm and the fraction volume 5 ml. Samples were dissolved in 6 M urea–1% mercaptoethanol before applying them to the column.

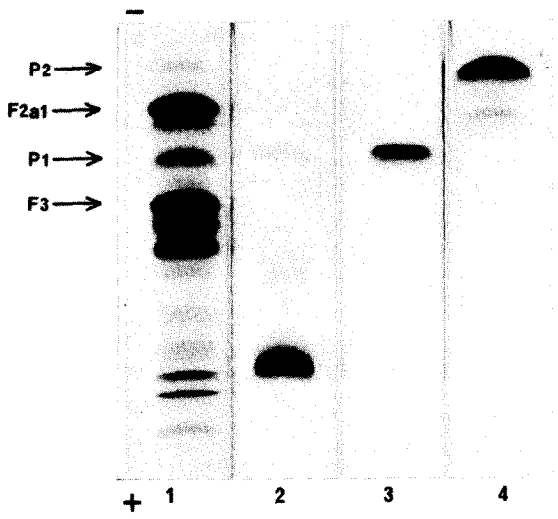

Fig.3. Electrophoretic pattern of histone degradation fragments from cycad pollen. Gel 1: mildly degraded total cycad histone; gel 2: the dimer of fragment P1 [10]; gel 3: fragment P1, and gel 4: fragment P2. All samples were dissolved in 6 M urea–1% mercaptoethanol prior to electrophoresis except 2 which was oxidized with iodosobenzoate [10].

The electrophoretic mobilities of a number of these fractions obtained from partially degraded nucleoprotein correspond to minor bands present also in histones isolated from calf thymus (see fig.6 in [11]) duck erythrocytes [11], chicken erythrocytes and rat liver [11]. This interesting constancy in the pattern of what may represent degradation products

Table 1
Amino acid composition of cycad Histone F3 and F2al and their degradation products

	Histone F3	Fragment P1	Expected* P1	Histone F2al	Fragment P2	Expected* P2
	mole %					
Lys	9.1	7.1	8.0	10.3	7.8	7.0
ε-*N*-MeLys	1.3	ND	?	trace	ND	?
His	1.7	1.6	1.8	2.2	1.8	2.6
Arg	13.4	14.0	12.5	14.8	13.8	16.3
Asp	3.8	4.9	4.5	4.7	7.1	5.8
Thr	6.5	4.8	5.3	5.7	6.6	8.2
Ser	3.2	3.0	4.5	1.7	3.6	1.3
Glu	11.3	12.3	11.6	6.8	9.0	7.0
Pro	4.6	4.7	4.5	1.9	2.9	1.3
Gly	5.6	4.5	4.5	16.0	10.4	10.4
Ala	14.5	13.1	13.4	7.3	8.2	7.0
$CySO_3H$	0.6	0.9	0.9	ND	ND	–
Val	4.7	5.2	5.3	7.3	6.0	9.3
Met	0.7	0.9	0.9	1.0	1.1	1.3
Ile	4.7	6.0	6.3	6.5	6.1	8.2
Leu	8.9	10.2	9.8	7.9	8.1	8.2
Tyr	1.6	2.1	1.8	3.8	4.0	5.1
Phe	3.8	4.5	4.5	2.1	2.8	2.6
NH_3	9.2	12.6	?	6.3	8.7	?

ND – Not determined.
No corrections for hydrolytic losses have been made.

* Calculated on the assumption that cycad histone F3 and F2al are identical to the pea histones [14,15]. Fragment P2 seemed to be slightly contaminated with a blocked serine proline rich fragment.

formed in a variety of different organisms together with the fact that partially degraded histones could be obtained from cycad in good yields prompted us to isolate and characterise two of these histone fragments.

We extracted degraded nucleoprotein with ethanolic HCl which is selective for arginine-rich histone [9]. From fig.1, gel 5, it is apparent that apart from the expected F3 and F2al histone two degradation products were extracted (P1 and P2). This protein mixture was subjected to exclusion chromatography on Biogel P-60 [12] (fig.2a). This yielded pure fragment P1 (fig.3, gel 3). It readily formed a dimer on oxidation [10] (fig.3, gel 2) indicating that it contained a cysteine residue. This fragment was subjected to amino acid analysis, and sequential degradation [13] on a Beckman 890 sequencer (table 1 and 2).

Fragment P2 co-eluted with undegraded histone F2al (fig.2a). Rechromatography of this fraction on the same Biogel P-60 column at higher salt concentration [12] resulted in selective aggregation of F2al (fig.2b) and thus the isolation of fairly pure fragment P2 (fig.3, gel 4). This fragment was then also subjected to amino acid and sequence analysis (table 1 and 2).

The sequence of the first 13 N-terminal amino acids in polypeptide P1 is identical to a sequence in histone F3 starting with residue 24 through 36. The

Fig.4. The N-terminal amino acid sequence of histone F3 [16] and F2al [15] around the proteolytic cleavage site (↓). Underlined residues represent the N-terminal sequence of cycad histone proteolytic fragments P1 and P2 (table 2). Lysine residues that may be modified to variable extents in various organisms [14–16] have been indicated. Histone F3 and F2al have been aligned to show maximum homology and to reveal the internal homology in histone F3 [16].

Table 2
Automatic sequential degradation [13] of proteolytic histone fragments from cycas

Fragment P1			Fragment P2		
Step no.	residue	yield in nmol	step no.	residue	yield in nmol
1	Ala	125	1	Arg*	112
2	Ala	90	2	His*	100
3	Arg*	56	3	Arg*	74
4	Lys*	16	4	Lys*	72
	Lys (Me)*	34		Lys (Me)*	trace
5	Ser	10	5	Val	50
6	Ala	54	6	Leu	41
7	Pro	42	7	Arg*	51
8	Ala	51	8	Asp*	24
9	Thr	20	9	Asp*	22
10	Gly	27	10	Ile*	28
11	Gly	23			
12	Val	48			
13	Lys*	30			

* Amino acids have been determined by amino acid analysis after re-hydrolysis of PTH-derivatives [13]. All other PTH-amino acids have been quantitated by gas chromatography [13]. The amount of fragments applied to the Beckman model 890 sequencer were 4 mg and 3 mg respectively.

sequence of the first 10 N-terminal amino acids in polypeptide P2 is identical to that of residue 17 to 26 in histone F2al (fig.4). In addition, the amino acid composition of the two fractions P1 and P2 corresponds to the calculated amino acid composition of polypeptides comprising the amino acid residues 24 through 135 of histone F3 and residues 17 through 102 of histone F2al respectively (table 2). From these data we conclude that fraction P1 and P2 are proteolytic degradation products derived from histone F3 and F2al respectively. The two histones must both have been cleaved at the C-terminal side of a lysine residue, histone F3 in position 23 and F2al in position 16. This indicates that the proteolytic enzyme performing these cleavages has a specificity similar to trypsin. It also becomes apparent that extensive homology near the cleavage site exists (fig.4), especially with regard to residues that may undergo modification through acetylation and methylation. It is well documented that modification of amino acids residues can alter the specificity of proteolytic enzymes, e.g. acetylation at lysine residues in a protein prevents the cleavage by trypsin at that site. The possibility thus exists that the susceptibility of histones to proteolytic degradation in chromatin could be regulated by the addition or removal of acetyl and methyl groups of lysine residues near the site of cleavage.

Modification of a lysine residue may either make the lysine residue unrecognizable for the enzyme thereby preventing a cleavage at that site, or may lead to a change in the interaction of histone with DNA or protein, changing the conformation of that region and making the histone accessible to the protease. The biological implication would be that the selective proteolytic cleavage of certain histones could provide a mechanism for the regulation of genes at the transcriptional or pre-transcriptional level. For example, the proteolytic cleavage of histones in highly packed chromatin could result in an unfolding of a particular region. Cleavage of histone F2al by the enzyme leads to the formation of a fragment (P2) which possesses drastically altered aggregation properties (fig.2a and b) as compared to

its parent protein resulting in a change of histone-histone interaction on the chromatin fibre with a possible alteration of the conformation of the latter. Minor polypeptide fractions corresponding in their electrophoretic mobility to the here characterized proteolytic degradation products of histone F3 and F2al also occur in histone fractions isolated from other animals [11]. This may indicate a wide distribution but unaltered specificity of the enzyme(s). It also becomes apparent that the rate of proteolytic degradation of F3 histone is very much greater when compared to that of histone F2al (e.g. fig.1).

The possible involvement of this proteolytic activity in transcriptional control can be deduced from experiments by Levitz et al. [17] which indicate that progesterone stimulates trypsin-like proteolytic activity in estradiol-primed rat uterus prior to an increase in template activity. In addition, Darzynkiewics et al. [18] have shown that reactivation of chick erythrocyte nuclei in heterokaryons is suppressed by inhibitors of trypsin and trypsin-like enzymes. Further work on the characterization of the enzyme is in progress.

Acknowledgements

We thank the C.S.I.R. (R.S.A.) and the University of Cape Town Research Committee for their support, and M. Morgan for expert technical assistance.

References

[1] Panyin, S., Jensen, R. H. and Chalkley, R. (1968) Biochim. Biophys. Acta 160, 252.
[2] Bartley, J. and Chalkley, R. (1970) J. Biol. Chem. 245, 4286–4292.
[3] Garrels, J. I., Elgin, S. C. and Bonner, J. (1972) Biophys. Res. Comm. 46, 454.
[4] Stellenwagen, R. H., Reid, B. R. and Cole, R. D. (1968) Biochim. Biophys. Acta 155, 581.
[5] Kurecki, T., Toczko, K. and Chmielewska, I. (1971) Bulletin de L'Académie Polonaise des Sciences 19, 627.
[6] Merkenschlager, M., Schlossmann, K. and Kurz, W. (1957) Biochemische Zeitschrift 329, 332.
[7] Brandt, W. F. and Von Holt, C. (1975) submitted for publication.
[8] Ohlenbusch, H. H., Olivera, B. M., Tuan, D. and Davidson, N. (1967) J. Mol. Biol. 25, 299.
[9] Johns, F. W., Phillips, D. M. P., Simson, P. and Butler, J. A. V. (1960) Biochem. J. 77, 631.
[10] Brandt, W. F. and Von Holt, C. (1971) FEBS Lett. 14, 338–342.
[11] Wangh, L., Ruiz-Carrillo, A. and Allfrey, V. G. (1972) Arch. Biochem. Biophys. 150, 44–56.
[12] Böhm, E. L., Strickland, W. N., Strickland, M., Thwaits, B. H., Van der Westhuyzen, D. R. and Von Holt, C. (1973) FEBS Lett. 34, 217–221.
[13] Brandt, W. F. and Von Holt, C. (1974) Eur. J. Biochem. 46, 419–429.
[14] Patthy, L., Johnson, J. and Smith, E. (1973) J. Biol. Chem. 248, 6834–6840.
[15] DeLange, R. J., Fambrough, D. M., Smith, E. L. and Bonner, J. (1969) J. Biol. Chem. 244, 5669.
[16] Brandt, W. F., Strickland, W. N., Morgan, M. and Von Holt, C. (1974) FEBS Lett. 40, 167–172.
[17] Levitz, M., Katz, J., Krone, P., Prochoroff, N. N. and Troll, W. (1974) Endocrinology 94, 663.
[18] Darzynkiewicz, Z., Chelmicka -Szorc, E. and Arnason, B. G. W. (1974) Proc. Natl. Acad. Sci. USA 71, 644–647.